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Metabolism of sulfamethoxazole in *Arabidopsis thaliana* cells and cucumber seedlings☆

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ABSTRACT

Reclaimed water is a historically underutilized resource. However, with increased population growth and global climate change, reclaimed water is evolving into an economical and sustainable water resource to meet the needs of citizens, industries, and agriculture. The use of recycled water for agricultural irrigation comes with the potential risk of environmental and food contamination by pharmaceuticals and personal care products (PPCPs). The levels of PPCPs in plants will depend on translocation and metabolism in plant tissues. However, relatively little is known about the metabolism of PPCPs in plants. In this study, the metabolism of the antibiotic sulfamethoxazole was investigated in *Arabidopsis thaliana* cells as well as cucumber seedlings grown under hydroponic conditions. Using high-resolution mass spectrometry and ^{14}C tracing allowed for sulfamethoxazole metabolism to be comprehensively characterized through all metabolic phases. Six phase I and II metabolites were identified in *A. thaliana* cell cultures and cucumber seedlings. Sulfamethoxazole metabolism followed oxidation and then rapid conjugation with glutathione and leucine. Direct conjugation with the parent compound was also observed via acetylation and glucosylation. At the end of 96 and 168 h incubation, N4-acetylsulfamethoxazole was the major metabolite and >50% of the radiolabeled sulfamethoxazole became non-extractable in both *A. thaliana* cells and cucumber seedlings suggesting extensive phase III metabolism and detoxification. The study findings provided information for a better understanding of the uptake and metabolism of sulfamethoxazole in higher plants, highlighting the need to consider metabolic intermediates and terminal fate when assessing the risk of PPCPs in the soil-plant continuum.

1. Introduction

Over the past two decades, pharmaceuticals and personal care products (PPCPs) have emerged as contaminants of environmental concern due to their extensive use and continuous emission into the environment (Daughton and Terns, 1999; Pedersen et al., 2005; Boxall et al., 2012). PPCPs are released into the environment primarily through the disposal of treated wastewater and biosolids from wastewater treatment plants (WWTPs) (Carballa et al., 2004). As climate change and population growth places an increasing stress on freshwater resources, especially in arid and semi-arid regions, communities have turned to utilizing municipal treated water for agricultural irrigation, which may result in soil contamination by PPCPs (Barnett et al., 2005; Tal, 2006; NRC, 2012). Furthermore, the heavy use of some pharmaceuticals, particularly antibiotics, for disease control and growth promotion in intensive animal farming also contributes to contamination of agricultural fields when animal wastes are used for fertilization (Hu et al., 2010).

The presence of PPCPs in irrigation water and soil can lead to contamination of food crops if plants can substantially accumulate these compounds. Various studies over the last decade have sought to quantify plant uptake of PPCPs, and in general, only low levels of PPCPs have been found in edible tissues (ng/kg) (e.g., Wu et al., 2013, 2014). The majority of studies to date have only targeted the parent form of PPCPs for analysis. However, plants have a cascade of enzymes that may extensively transform xenobiotics such as PPCPs after uptake (Celiz et al., 2009; Fu et al., 2016). Recently several published studies have explored the metabolism of pharmaceuticals in plants (e.g. Huber et al., 2009, 2012; Fu et al.,

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2016; LeFevre et al., 2016; Marsik et al., 2017). Therefore, consideration of metabolism and biologically active metabolites is much needed for a better understanding of the fate and risks of PPCPs in the soil-plant system.

Higher plants have many detoxification enzymes similar to those in animals. These enzymes function in plants as a 'green liver' (Sandermann, 1994). In general, metabolism of xenobiotics includes three phases. Phase I involves modification reactions such as oxidation, hydrolysis, and dealkylation reactions introducing reactive sites to the molecule. Phase II is characterized by conjugation with large polar biomolecules, such as sugars and amino acids, to further increase the polarity of the xenobiotic. Phase III is typified by sequestration, resulting in the formation of bound residues (Sandermann, 1992; Sandermann, 1994, Miller et al., 2016). As shown for many xenobiotics in mammals and plants metabolites from phases I and II often retain biological activity (Osborne et al., 1990; Pichersky and Gang, 2000; Miller et al., 2016), and therefore should not be discounted.

In this study, sulfamethoxazole was selected as the compound of interest because of its prevalence in WWTP effluents and increasing concerns over the propagation of antibiotic resistance (Yao et al., 2012; WHO, 2016). Since its introduction in 1961 sulfamethoxazole has been widely prescribed due to its potency against both gram-positive and gram-negative bacteria (Brunton et al., 2011). Currently, sulfamethoxazole's has been detected from ng L^{-1} to μg L^{-1} in surface and effluent waters and μg kg^{-1} to mg kg^{-1} in soils and manure (Hu et al., 2010; Shelver et al., 2010; Brausch et al., 2012). Recent long-term studies of waste-water application under realistic field conditions have highlighted the potential for sulfamethoxazole to be taken up and translocated in crop plants, including to the fruit (Christou et al., 2017).

The structures of sulfamethoxazole metabolites, including conjugates from Phase II metabolism, were identified using high-performance liquid chromatography coupled with time-of-flight high-resolution mass spectrometry (HPLC-TOF-HRMS) and further quantified using ultra-high performance liquid chromatography in tandem with a triple quadrupole mass spectrometry (UPLC-TQD-MS/MS). Furthermore, Phase III terminal products in the form of bound residues were quantified using ^{14}C labeling.

Arabidopsis thaliana cells were selected as the experimental organism due to their extensive use in the literature, commercial availability, and their membership in the commonly consumed Brassica family (e.g., cabbage, broccoli, kale). Further, *Arabidopsis thaliana* plants are found worldwide under several common names (e.g., Wall cress, mouse-ear cress, shiro-inu-nazuna) and are consumed by a wide variety of animals as well as humans (van Poecke and Dicke, 2004, TAIR institute, 2018). Cucumber (*Cucumis sativus*) was selected in the hydroponic experiment due to the fact that it is often consumed raw, rapid growth, and amiability to soilless culture (Texas A&M, AgriLife, 2018).

2. Materials and methods

2.1. Chemicals and solvents

Non-labeled sulfamethoxazole was purchased from MP Biomedicals (Solon, OH). Sulfamethoxazole-d_4 was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and ^{14}C-labeled sulfamethoxazole was obtained from American Radiolabeled Chemicals (Saint Louis, MO). Stock solutions of ^{14}C-sulfamethoxazole and non-labeled sulfamethoxazole were prepared in methanol to reach a specific radioactivity of 1.2×10^3 dpm μL^{-1} and a chemical concentration of 1.0 mg mL^{-1}, respectively. HPLC grade acetonitrile and methanol were used for extraction along with ultra pure water. Mobile phases were prepared using Optima™ LC/MS grade methanol and deionized water. Standards were prepared in HPLC grade methanol and stored in the dark at −20 °C. All solvents used in this study were purchased from Fisher (Fair Lawn, NJ).

2.2. Arabidopsis thaliana cell incubation experiment

PSB-D *A. thaliana* cell line (CL84840) was purchased from the Arabidopsis Biological Resource Center (ARBC) at the Ohio State University (Columbus, OH). The cells were maintained in liquid suspension culture at 25 °C and rotated at 130 rpm in the dark according to the ARBC protocol (2018). To explore metabolism of sulfamethoxazole in *A. thaliana* cells, 7 mL of cell culture was inoculated in 43 mL fresh culture and cultivated for 96 h at 25 °C and 130 rpm in the dark to produce the seed culture. A 30 μL aliquot of the non-labeled stock solution and 10 μL aliquot of ^{14}C-sulfamethoxazole were spiked into 30 mL of *A. thaliana* cell culture, resulting in a nominal initial concentration of sulfamethoxazole of 1 μg mL^{-1} and a specific radioactivity of 1.2×10^3 dpm mL^{-1} (0.3% methanol). Simultaneously, control treatments were prepared by autoclaving cell suspensions before chemical spiking (non-viable cell control), flasks containing sulfamethoxazole without cells (medium control), and flasks containing live cells but no sulfamethoxazole (background control). These control treatments were used to determine adsorption, abiotic degradation, and potential toxicity to cells. The incubation lasted for 96 h, and triplicate containers were sacrificed at 0, 3, 6, 12, 24, 48 and 96 h.

At each sampling interval, the entire culture was transferred to a 50 mL polypropylene centrifuge tube and centrifuged at 10,000 rpm for 15 min. The supernatant was collected and stored at −20 °C until further analysis and the plant cells were placed at −80 °C before freeze-drying for 72 h. After drying, cells were fortified with 50 μL of 10 mg L^{-1} sulfamethoxazole-d_4 as a recovery surrogate. Cells were extracted using a modified method previously established in Wu et al. (2012). Briefly, cells are sonicated in a Fisher Scientific FS110H sonication bath (50/60 Hz, Pittsburgh, PA) for 20 min with 30 mL acidified DI water (pH 4) followed by centrifugation at 10,000 rpm for 15 min. The supernatant was decanted into a new 50 mL centrifuge tubes. The cell matter was further extracted using 20 mL methyl tert-butyl ether (MTBE), followed by 20 mL acetonitrile. The MTBE and acetonitrile supernatants were combined, dried under nitrogen at 35 °C, and re-constituted in 1.0 mL methanol. The extract was then combined with the above water extract. The combined sample extract was loaded onto a preconditioned 150-mg Oasis© HLB solid phase extraction (SPE) cartridge and eluted with 20 mL methanol. The cleaned extract was dried under nitrogen and further recovered in 1.5 mL 50:50 MeOH:H_2O (v/v). The growth media was acidified to pH 3 and similarly extracted and cleaned as described above. Extraction recovery for the sample preparation protocol of the cell extract was 46% ± 13 and for the growth media was 57% ± 10.

Prior to instrument analysis, both cell and media extracts were transferred to micro-centrifuge tubes and centrifuged at 120,000 rpm in a bench-top SciLogex d2012 centrifuge (Rocky Kill, CT) and further filtered through a 0.22-μm polytetrafluoroethylene (PTFE) membrane (Millipore, Carrigtwohill, Cork, Ireland) into 2 mL glass vials. All final extracts in 2 mL glass vials were stored at −20 °C if not immediately analyzed.

At each time interval, 100 μL of the cell material extract or concentrated growth media was added to 6 mL Ultima Gold™ liquid scintillation cocktail (Waltham, MA) to measure the extractable ^{14}C-radioactivity on a Beckman LS 5000TD Liquid Scintillation Counter (LSC, Beckman, Fullerton, CA). Additionally, the extracted cell matter was air dried, and a 10 mg aliquot was combusted on an OX-500 Biological Oxidizer (R. J. Harvey Instruments, Hillsdale, NJ). The evolved $^{14}CO_2$ was captured in 15 mL

Harvey Carbon-14 cocktail II and the ^{14}C activity was measured to derive the fraction of bound residues from Phase III metabolism.

2.3. Whole plant hydroponic cultivation experiment

Uptake and metabolism of sulfamethoxazole were further evaluated using whole cucumber seedlings. Cucumber seeds were purchased from Fisher (Fairfield, NJ). Seedlings were started in a commercially purchased organic soil (Parkview Nursery Riverside, CA) in a growth chamber (22 °C 16 h day/20 °C 8 h night cycle; relative humidity of 75–80%). After the appearance of the first true leaves, plants of uniform size were selectively removed from pots, rinsed with DI water and placed in 1 L amber glass jars containing hydroponic solution (Oasis® 16-4-17 hydroponic fertilizer 3.16 g L^{-1}). After acclimating for 4 d, each jar was treated with 100 μL of non-labeled sulfamethoxazole stock solution to arrive at a nominal concentration of 1 μg L^{-1}. Simultaneously, 7.6 μL of ^{14}C-sulfamethoxazole stock solution was added to reach an initial specific radioactivity of 8.6×10^3 dpm mL^{-1}. Jars containing seedlings without sulfamethoxazole and jars containing sulfamethoxazole but no plant were similarly prepared as controls. After 7 d, seedlings were removed from the jars, and their roots were carefully rinsed with DI water and dried with paper towels. Roots, stems, old leaves (those present before the start of incubation) and new leaves (those that grew during the incubation) were separated from each other using a razor blade. The separated tissues were stored at −80 °C until analysis.

Plant tissues were placed in a freeze-drier for 72 h, and a 0.2 g aliquot of the dried plant material was ground in liquid nitrogen using a mortar and pestle. The pulverized tissue samples were subsequently extracted and cleaned as described above for *A. thaliana* cells. The hydroponic media was collected, filtered with GF/F filters, acidified to pH 3 with HCL, and extracted using an HLB cartridge as previously described.

2.4. Structural elucidation using mass spectrometry

Instrument analysis was first performed on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) in tandem with a Waters Micromass triple quadrupole (TQD) mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters, Milford, MA) to determine the rate of uptake and the concentration of the parent compound. Separation was achieved on an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 μm, Waters) at 40 °C. Mobile phase A consisted of water containing 5% methanol and acidified using 0.001% formic acid. Mobile phase B was composed of pure methanol. The solvent gradient program, in reference of mobile phase A, was as follows, 0–2 min 95%; 2–3min 30%; 3–3.25 min 5%; 3.25–4.5 95%. The flow rate was 0.3 mL min^{-1} and the injection volume was 5 μL. The mass data were acquired using Intellistart® (Waters) in multiple reactions monitoring (MRM) and in the positive ESI mode. Proposed metabolites were scanned in selective ion reaction (SIR). Mobile phase A and B consisted of acidified water and methanol (0.1% formic acid) respectively. The solvent gradient program, in reference of mobile phase A, was as follows, 0–5 min 95%, 5–9 min 50%, 9–10 min 0%, 10–12 min 95%. The flow rate was 0.4 mL and the injection volume was 5 μL. The specific instrument settings were: capillary voltage 1.7 kV, collision gas (Aragon, 99.9%), dwell time 0.022 s, source temperature 150 °C, desolvation temperature 450 °C, desolvation gas 900 L h^{-1} and cone gas 50 L h^{-1}. The cone voltage (V) and the collision energy (V) for each standard can be found in Table S1.

High resolution LCMS data were obtained using an Agilent 1200 series HPLC (with UV detector) coupled to an Agilent 6210 time-of-flight high resolution mass spectrometer (TOF-HRMS) with ESI/APCI mixed ion source. The separation was achieved on a Thermo Scientific Hypersil Gold C18 column (2.1 mm × 100 mm, 3 μm). Mobile phase A consisted of water containing 0.1% formic acid. Mobile phase B was composed of acetonitrile 0.1% formic acid. The solvent gradient ran from 5% to 100% B in 18 min at 0.3 mL min^{-1} flow rate. Samples were analyzed at the High Resolution Mass Spectrometry Facility in the Chemistry Department at the University of California, Riverside. Raw data files were obtained and converted to mzXMLfiles using ProteoWizard MSConvert and analyzed using MZmine 2 open software (Pluskal et al., 2010). Candidate metabolites were proposed based on the presence of unique peaks in the treatment that were absent in the controls (Figure S1). Identification uncertainty was determined using the Warwick mass accuracy calculator by comparing theoretical m/z to the observed m/z.

To structurally identify sulfamethoxazole metabolites, the uncertainties in confirmation were evaluated against the metabolite identification criteria as outlined in Schymanski et al. (2014a,b). Using the criterion, Level 1 structures are those with direct confirmation against authentic standards. Level 2 structures are probable structures based on library spectrum data, literature data, and experimental information and Level 3 structures are tentative structures derived from strong MS/MS information for the proposed structures, but the position of substitutions could not be determined with certainty (Table 1). Structural identification for sulfamethoxazole metabolites was determined from accurate mass information and fragmentation patterns received using the QTOF mass analyzer (Figure S2). The information was compared to mass spectra libraries and/or the literature on known human metabolites of sulfamethoxazole. Further, identified metabolites were scanned in the selective ion reaction, multiple reaction monitoring and MS scan modes using Targetlynx™ software (Waters) with comparison against authentic standards when available (Figure S3). Results were further compared with literature reporting common enzymes in the metabolism of other xenobiotics in plants (Vanderford and Snyder, 2006; Badenhorst et al., 2013; Roa, 2015) to determine the most likely pathways and metabolite structures of sulfamethoxazole.

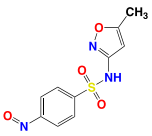
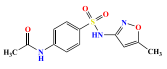
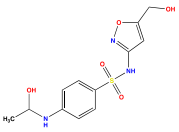
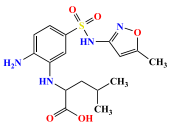
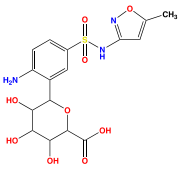
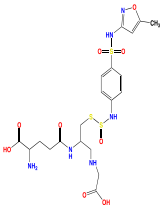
The relative fractions of individual metabolites were determined on the Waters UPLC-TQD MS/MS in the selective ion reaction scan mode. Authentic standards of sulfamethoxazole and N4-acetylsulfamethoxazole (S296) were used as an example to verify the identity of proposed structures as well as to quantify these compounds. Retention times, accurate mass, and fragmentation patterns were used for validation.

2.5. Data analysis and quality control

Individual peaks were detected and integrated using TargetLynx XS software (Waters). Data were analyzed and graphed using the Prism 6 GraphPad software (La Jolla, CA). Results were calculated as the mean ± standard error (SE), and a Student's t-test was conducted to assess systematic differences between multiple groups and two groups ($\alpha = 0.05$).

All treatments in the *A. thaliana* cell incubation experiment were conducted in triplicate, and all treatments in the hydroponic cultivation experiment were conducted in quadruple (to account for potential loss of plants). Calibration curves were prepared with standards of sulfamethoxazole, sulfamethoxazole-d_4 and N4-acetylsulfamethoxazole and the linear range with $r^2 \geq 0.99$ was used to ensure accuracy. A limit of detection of 3 ng mL^{-1} and a limit of quantification of 5 ng mL^{-1} for sulfamethoxazole were determined based on a signal to noise ratio of 3 and 10, respectively. Whenever possible, authentic standards, online mass spectra databases, and mz calculators were used to verify the identity of the proposed metabolic products.

Table 1
Summary of the mass spectra information and proposed structures of sulfamethoxazole and its metabolites.

Name	ID	t_r (min)	Obs. m/z	Calc. m/z	Mass difference	Predicted formula	Fragments (m/z)	Structure Proposed	Confidence level[2]
Sulfamethoxazole[1]	SMX	10.57	254.0552	254.0521	±0.002	$C_{10}H_{11}N_3O_3S$	$C_6H_5NO_2S$ 155.9934 C_6H_8NS 127.0593 C_6H_4S 108.0362		Level 1 AS, HR-MS, MRM
Nitroso- Sulfamethoxazole[2]	S268	2.06	268.1158	268.1392	±0.02	$C_{10}H_9N_3O_4S$	$C_{10}H_9N_3O_3S$ 252.1170 $C_{10}H_6N_3OS$ 216.2067 $C_6H_2NO_2S$ 152.0517 $C_4H_4N_2OS$ 132.1073		Level 2b HR-MS, SIR (Bovin et al., 2013)
N4- acetylsulfamethoxazole[3]	S296	11.60	296.0751	296.0705	±0.004	$C_{12}H_{13}N_3O_4S$	-OH 279.1728 $C_8H_8N_2OS$ 180.1797 C_8H_8NOS 163.1293 $C_4H_4N_2OS$ 129.0783		Level 1 AS, HR-MS, MRM
N4-Acetyl-5-OH– sulfamethoxazole[4]	S313	4.76	313.0781	313.0732	±0.004	$C_{12}H_{14}N_3O_5S$	$-CH_3$ 299.2023 $-CH_5O$ 279.1739 $C_{10}H_{11}N_3O_3S$ 254.0648 $C_7H_4N_2O_3S$ 194.1178 C_6H_5OS 124.0893		Level 2b HR-MS, SIR (Zhou et al., 2009)
C3-leucyl-sulfamethoxazole[5]	S385	15.49	385.1852	385.1546	±0.031	$C_{16}H_{24}N_4O_5S$	$-C_3H_8O$ 325.2122 $-C_2H_{12}O_3$ 301.1429 $C_{12}H_{15}N_2O_4S$ 283.1326 $C_{10}H_{13}N_3O_3S$ 255.1677 $C_{12}H_{18}O_2N_2$ 223.1135 C_6H_5OS 124.0897		Level 3 HR-MS, SIR
Sulfamethoxazole-glucuronide[6]	S430	6.76	430.0893	430.0836	±0.006	$C_{16}H_{19}N_3O_9S$	$C_{15}H_{20}N_3O_9S$ 419.3306 $C_{16}H_{13}N_3O_7S$ 391.3031 $C_{12}H_{14}NO_7S$ 316.2214 $C_{10}H_9N_3O_3S$ 251.1917		Level 2b HR-MS, SIR (Rieder, 1973)
N4-sulfsulfamethoxazole-glutathione conjugate[7]	S606	16.71	606.0894	606.0951	±0.006	$C_{20}H_{25}N_6O_{11}S_3$	$-CH_7O_2$ 555.3787 $C_{16}H_{20}N_4O_7S_3$ 476.3320 $C_{12}H_{16}N_4O_5S_3$ 391.3031 $C_{10}H_{12}N_3O_4S_2$ 302.1659 $C_{10}H_{13}N_3O_3S$ 255.2408 C_6H_5OS 124.0893		Level 2b HR-MS, SIR (Cribb et al., 1991)

AS: confirmed by authentic standard; MRM: Multiple Reaction Monitoring; SIR: Selective Ion Reaction MSL: Comparison with mass spectra library; HR-MS: High resolution mass spectrometry; t_r retention time obtained from HPLC TOF-MS/MS analysis.
[1]4-Amino-N-(5-methyl-1,2-oxazole-3-yl)benzenesulfonamide), [2]N-(5-Methyl-3-isoxazolyl)-4-nitrobenzenesulfonamide, [3]N-{4-[5-Methyl-1,2-oxazol-3-yl)sulfamoyl] phenyl}ethanimidic acid, [4] N-(4-{[5-(hydroxymethyl)-1,2-oxazol-3-yl]sulfamoyl}phenyl)ethanimidic acid, [5]2-[2-Amino-5-(5-methyl-3-isoxazolylaminosulfonyl)phenyl-amino]-4-methylvaleric acid, [6]N-(5-Methylisoxazole-3-yl)-N-(beta-D-glucopyranuronosyl)-4-aminobenzenesulfonamide, [7]2-amino-4-{[(1R)-1-[(carboxymethyl)carba-moyl]-2-{[({4-[(5-methyl-1,2-oxazol-3-yl)sulfamoyl]phenyl}amino)sulfinyl]sulfanyl}ethyl]carbamoyl}butanoic acid.
[a]Schymanski E. et al., 2014a,b.

3. Results and discussion

3.1. Kinetics of parent, extractable and non-extractable residues

The metabolism of sulfamethoxazole in *Arabidopsis thaliana* cells was validated using a range of controls. No sulfamethoxazole was detected in the media or cell blanks, and there was no detectable disappearance of sulfamethoxazole in the cell-free media, suggesting the absence of contamination or abiotic transformation. Moreover, no significant difference was seen in the cell mass between the chemical-free control and the treatments indicating that sulfamethoxazole did not affect the growth of *A. thaliana* under the experimental conditions. In the non-viable cell control, it was found that sulfamethoxazole was adsorbed to the cell matter, but the fraction did not contribute significantly to the dissipation of sulfamethoxazole from the media ($P > 0.05$). In contrast, in the live cell treatments, sulfamethoxazole dissipated appreciably from the media, with the average concentration decreasing from 246 ± 1.74 ng mL^{-1} initially to 176 ± 5.23 ng mL^{-1} after 96 h of incubation (Fig. 1). Concentrations were therefore not adjusted for recovery or loss to adsorption to cell matter or the surfaces of the flask. As there was no significant difference between measured initial concentrations of the cell-free flasks, and viable and non-viable flasks, we assumed that adsorption to the container was insignificant.

Concurrent to the dissipation in the medium, sulfamethoxazole was detected in the *A. thaliana* cells, and the level was the highest at 3 h sampling point decreasing thereafter. The presence of sulfamethoxazole in the live cells provided direct evidence of its uptake into *A. thaliana* cells. The level of sulfamethoxazole in the cell matter decreased after reaching the maxima at 3 h, Fitting a decrease in the sulfamethoxazole level in the cell to a first-order decay model yielded a half-life of 19.4 h ($r^2 = 0.94$). This was in comparison to a biological half-life of 10 h in humans for sulfamethoxazole (Law et al., 2014). The decrease of sulfamethoxazole in the live *A. thaliana* cells suggested active metabolism.

The use of ^{14}C labeled sulfamethoxazole enabled determination of the fractions of sulfamethoxazole and its metabolites that were incorporated into the cell matter, which could not be characterized using traditional extraction and analytical methods. A rapid increase in the bound residue fraction was observed during the 96 h cell cultivation, while the increase in the extractable residue form in the cells was more gradual (Fig. 2). During the incubation, the fraction in bound residues increased steadily to $53 \pm 10\%$ at 96 h, clearly suggesting that *A. thaliana* cells were capable of effectively metabolizing and then sequestering sulfamethoxazole and its metabolites in the cell system. In contrast, the fraction of the extractable residues was relatively low, ranging from 0% to $22 \pm 1.6\%$. Extractable residues in plant metabolism are thought to contain Phase I and Phase II metabolites, including conjugates, while Phase III metabolism results in the incorporation or sequestration of metabolites into the cell wall (Sandermann, 1994). Therefore, formation of bound residues may be regarded as detoxification of a xenobiotic in plants. Several previous studies also demonstrated that plant cells and whole plants were capable of metabolizing PPCPs, transforming them into more polar intermediates and sequestering them in their cell walls or vacuoles (Huber et al., 2009, 2012; Fu et al., 2016).

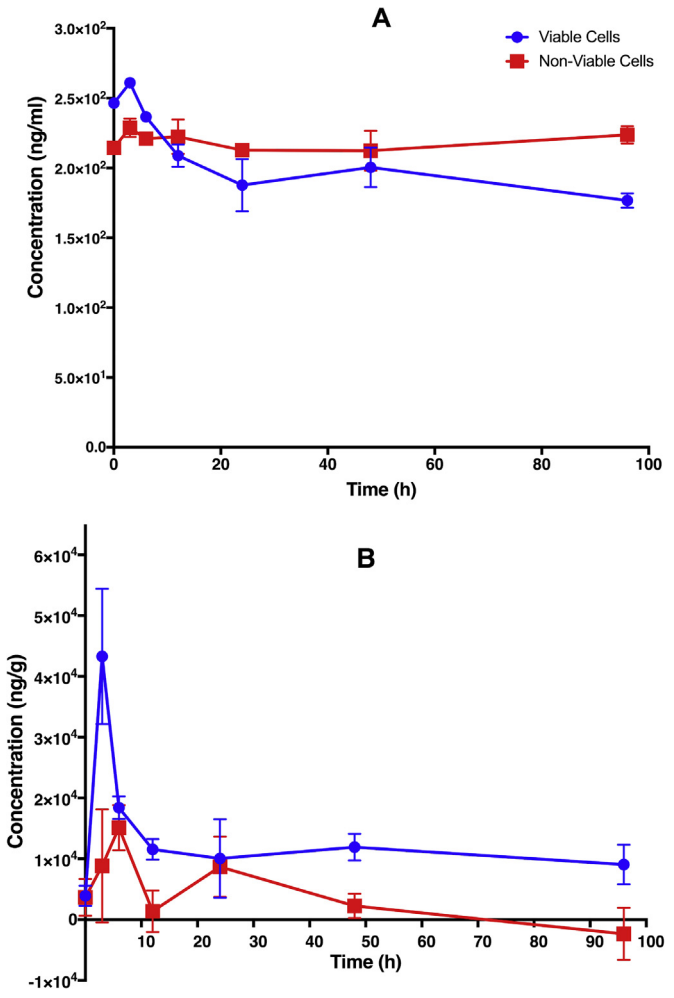


Fig. 1. Sulfamethoxazole in the medium and *Arabidopsis thaliana* cells. (A) Kinetics of sulfamethoxazole in cell growth media. (B) Kinetics of sulfamethoxazole in viable and nonviable cells. Error bars represent the standard error of triplicate.

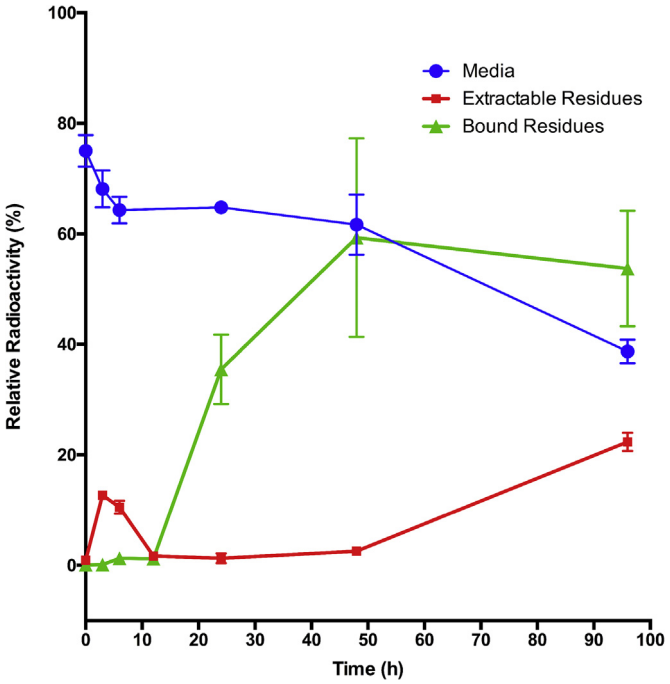


Fig. 2. Distribution of ^{14}C activity in media and *Arabidopsis thaliana* cells after treatment with ^{14}C-sulfamethoxazole (nominal % of initially spiked radioactivity).

3.2. Metabolism of sulfamethoxazole in A. thaliana cells

In *A. thaliana* cells, tentative metabolism pathways of sulfamethoxazole were derived by combining spectra data, knowledge of human metabolism of sulfamethoxazole and its degradation in water systems (Table 1 and Fig. 4). In the proposed metabolism pathways, sulfamethoxazole underwent Phase I metabolism including oxidation and hydroxylation reactions, which was followed by Phase II metabolism through acetylation and rapid conjugation with glucuronic acid, amino acids, and glutathione (Figs. 3 and 4). The end products of Phase II metabolism were then further sequestered likely through incorporation into cell walls and other cell components, resulting in the formation of non-extractable bound residues.

4-Nitroso-sulfamethoxazole (designated as S268) is a known metabolite in human metabolism. The knowledge of its formation

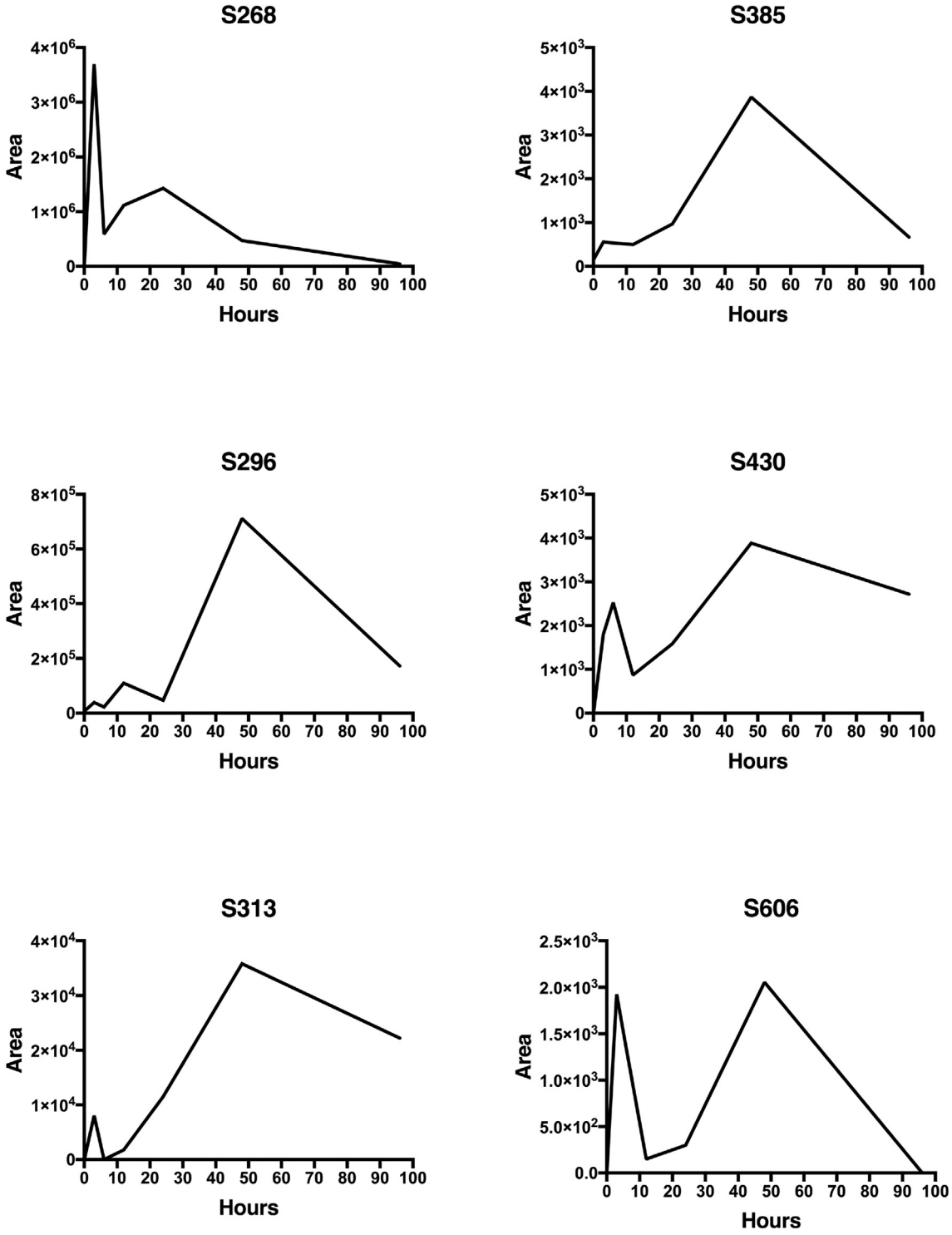


Fig. 3. Kinetics of proposed sulfamethoxazole metabolites in *Arabidopsis thaliana* cells (based on peak area).

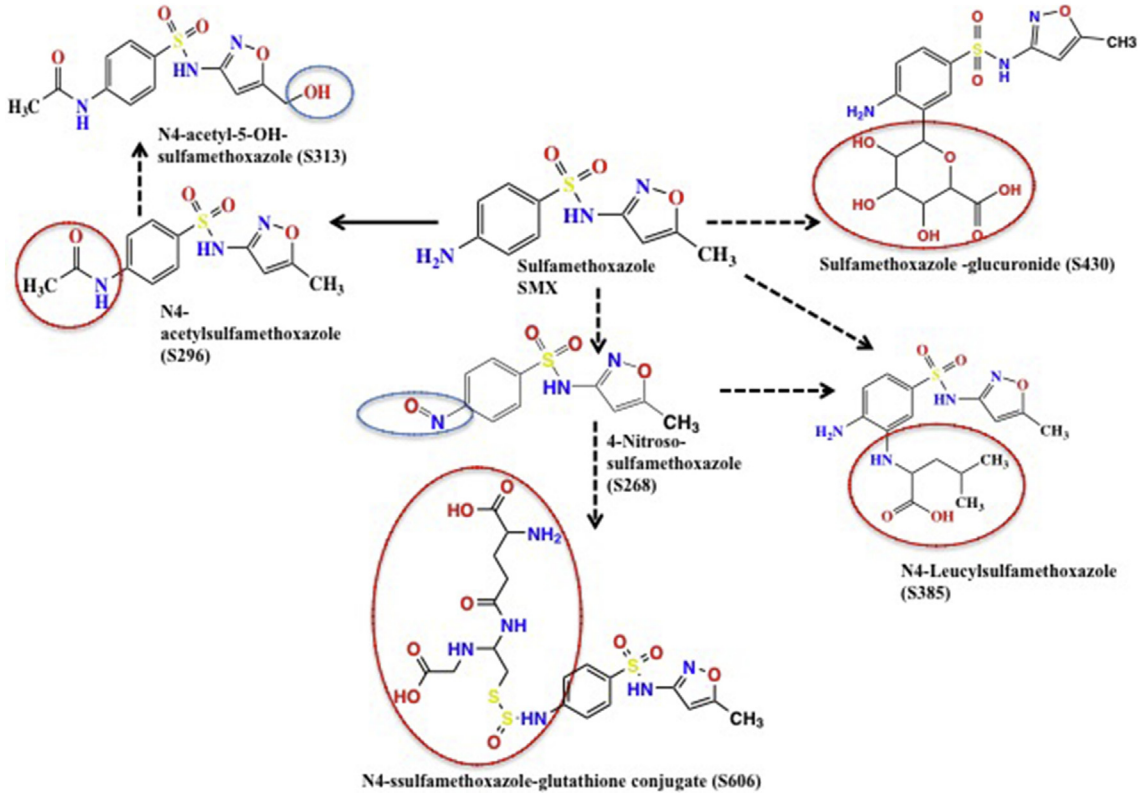


Fig. 4. Proposed metabolism pathways of sulfamethoxazole in *Arabidopsis thaliana* cells. Blue (Phase I) and Red (Phase II) circles represent the altered functional group. Dashed arow: Proposed structures; solid arrow:Verified structured. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in mammalian livers, structure, and properties (Cribb et al., 1991) was used to detect its presence in the HPLC-TOF MS scans. Subsequent UPLC-MS/MS scans suggested the rapid formation of this intermediate. Individuals with low production of *N*-acetyltransferase were previously reported to exhibit a high production of hydroxylamine sulfamethoxazole and nitroso-sulfamethoxazole. In humans, these metabolites were found to be responsible for the adverse side effects associated with the consumption of sulfonamides, such as skin rashes or hives (Cribb et al., 1993). N4-Acetyl-5-OH-sulfamethoxazole (S313) was also previously shown to form by cytochrome-P450 oxidation in mammalian livers (Zhou et al., 2009). In higher plants, it was likely formed through oxidation reactions mediated by cytochrome-P450 enzymes, a superfamily of enzymes in both plants and mammals (Gonzalez and Nebert, 1990).

N4-Acetylsulfamethoxazole (S296), sulfamethoxazole-glucuronide (S430) and N4-sulfamethoxazole-glutathione conjugate (S606) metabolites were all previously found in human metabolism of sulfamethoxazole (van der Ven et al., 1995). N4-acetylsulfamethoxazole was detected in *A. thaliana* cells and confirmed using its authentic standard. The glucose and glutathione conjugates were detected by comparing the exact mass and fragmentation patterns to proposed spectra libraries for each compound (TMIC, 2017). However, observed difference in the fragmentation patterns indicated that conjugation location differed from in those observed for human metabolism. A similar pattern was observed in previous studies concerning the plant metabolism of pharmaceuticals (Fu et al., 2017).

The proposed amino acid conjugate in *A. thaliana* cells is, to the best of our knowledge, the first evidence for their occurrence in higher plants. Conjugation with amino acids has been considered a detoxification pathway for other pharmaceuticals (Fu et al., 2016; Marsik et al., 2017). The structure proposed here for leucyl-sulfamethoxazole (S385) was, in part, based on a (M + H) m/z of 132.0765, 223.1135 and 255.1677 showing distinct fragments of $C_6H_{13}NO_2$ $C_{12}H_{18}N_2$ and $C_{10}H_{12}N_3O_3S$ (Table S2). The position of the amino acid on the benzene ring was selected based on optimum stable formation (Paulson et al., 1986; Carney and Giuliano, 2011).

In the tentative metabolism pathways in *A. thaliana* cells, sulfamethoxazole underwent Phase I oxidation forming 4-nitroso-sulfamethoxazole (S268) which was followed by phase II conjugation with leucine or glutathione. Based on the signal strength, a relatively high level of the *N*5-leucyl-sulfamethoxazole conjugate (S385) was detected at the 3 h sampling point but remained at trace levels for most of the incubation duration, with the exception of the 48 h sampling point. The glutathione conjugate (S606) appeared quickly (3 h into incubation), spiked at 48 h, and decreased to a non-detectable level by the end of the cultivation (Fig. 3).

Conjugation with glucuronic acid (S430) was also observed to form quickly (3 h), and direct glycosylation of sulfamethoxazole has also been observed in mammals (van der Ven et al., 1995). Another pathway appeared to be acetylation of the sulfamethoxazole amine followed by rapid oxidation to form the S296 and S313 metabolites (Fig. 3), with N4-acetylsulfamethoxazole being the predominant metabolite at the end of incubation. It has been shown that this acetylation pathway predominates in human metabolism for detoxification (Spielberg, 1996a,b). Future research should be conducted to determine if enzymes similar to those seen in human metabolism of sulfamethoxazole actively participate in its metabolism in plants, such as CYP2C9 and *N*-acetyltransferases 1 and 2.

Table 2
Detection of ^{14}C-sulfamethoxazole in cucumber seedlings after 7 d growth in nutrient solution (nominal % of spiked ^{14}C activity).

	Extractable Residues (%)	Bound Residues (%)	Parent Compound (ng/g)	Parent Compound (%)
Roots	1.09 ± 0.38	20.93 ± 2.80	3155 ± 1439	1.06 ± 0.40
Stems	0.91 ± 0.17	1.83 ± 0.51	2486 ± 202.2	0.52 ± 0.29
Old Leaves	1.56 ± 0.04	2.95 ± 0.85	2780 ± 1341	0.82 ± 0.39
New Leaves	1.67 ± 0.11	1.48 ± 0.43	N.D.	N.D
Media	56.02 ± 3.40		4658 ± 1626	46.6 ± 16.3

N.D. = Not detected.

3.3. Uptake, translocation and transformation in cucumber seedlings

Similar metabolites were found in cucumber seedlings grown in the nutrient solution containing sulfamethoxazole. Metabolites from Phase I and Phase II metabolism were similarly detected and extensive Phase III sequestration was further observed (Table 2). When cucumber plants were exposed to 1.0 μg L^{-1} sulfamethoxazole in nutrient solution, sulfamethoxazole was taken up into the plant, with accumulation primarily in the roots, old leaves, and

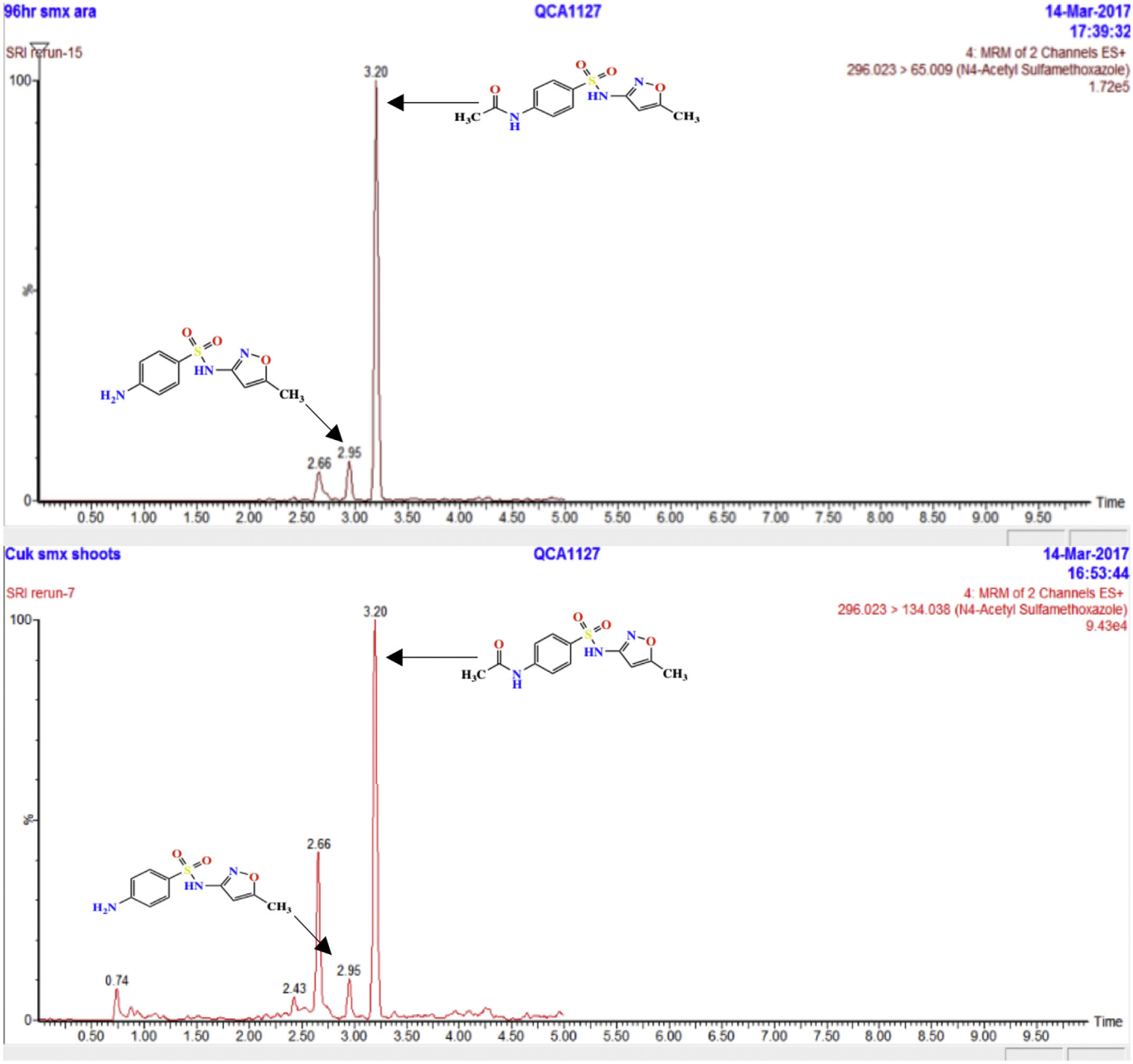


Fig. 5. Representative chromatogram of the metabolite N4- acetylsulfamethoxazole in cucumber shoots and *Arabidopsis thaliana* cells.

stems, and absent in the new leaves (Table 2). When ^{14}C activity was used for calculation, a translocation factor (TF), i.e., the ratio of the concentration in leaves/stems over that in the roots, was estimated to be 0.32. While this value was higher than that reported by Dodgen et al. (2015) (based on the parent form) it suggested that sulfamethoxazole is not readily translocated in the plant after entering the root. This was consistent with models used to predict the behavior, e.g., diffusion through cell membranes, of similar pharmaceutical compounds (Trapp et al., 2009). These models suggested that polar compounds (log K_{ow} 0 to 1), such as sulfamethoxazole, would have translocation factors from the solution to the xylem ranging from 0.25 to 0.5.

Bioconcentration factors were calculated for the roots (BCF_R) and the shoots (BCF_S) as the ratio of specific radioactivity in the tissue over that in the growth media. The mean BCF_R and BCF_S were determined to be 1.59 and 0.53, respectively. The calculated BCF_R and BCF_s were similar to those found for other vegetable species in Dodgen et al. (2015). These studies together suggested that while plants are capable of taking up sulfamethoxazole, it mainly remains in the root with limited potential for translocation to the other organs of the plant (Wu et al., 2012, 2015; Miller et al., 2016).

After the 7 d cultivation, sulfamethoxazole parent and metabolites identified in the *A. thaliana* cell incubation were similarly scanned in cucumber samples from the hydroponic cultivation experiment, including plants tissues and blank control without plants. The proposed metabolites were not detected in the control treatment or the cucumber hydroponic solution, indicating that the transformation occurred within the cucumber seedlings following uptake. All of the metabolites proposed for *A. thaliana* cells were detected in the cucumber seedlings. However, due to the lack of authentic standards for most of the metabolites or low signal (S/N < 3), we did not attempt to quantify individual metabolites in the cucumber plants (Fig. 5). In a previous study, Chen et al. (2017) investigated the uptake, metabolism, and elimination of sulfamethoxazole *in Brassica rapa chinensis* and *Ipomoea aquatica*. In that study, no metabolites of sulfamethoxazole were detected in plant tissue. This could be attributed to a number of factors, such as, differences in extraction protocols, instrument analysis or extensive phase III metabolism that decreased the level of metabolites below the limit of detection. Our findings were in line with previous research conducted with the related sulfa-drug sulfamethazine, in which N-acetyl-sulfamethoxazole and hydroxy-sulfamethoxazole were detected in *Zea mays* L. plants (Michelini et al., 2012).

The total amount of extractable and non-extractable residues in the cucumber plants ranged from 94% to 80% indicating that some mineralization (conversion to CO_2) occurred. This rate of mineralization in the plant cultivation system was higher than that of sulfamethoxazole in soils (Holtge and Kreuzig, 2007). Because plant respiration (the release of CO_2) may contribute to the loss of 14C after mineralization, this finding further highlights the detoxification prowess of higher plants (Dodgen et al., 2014).

4. Conclusions

Results from this study demonstrated that ^{14}C tracing, high-resolution mass spectrometry, and cell and hydroponic cultures may be used in a complementary manner to obtain a complete depiction of plant metabolism of emerging contaminants. The antibiotic sulfamethoxazole was found to be taken up and metabolized extensively by *A. thaliana* cells and cucumber seedlings. The Phase I metabolism involved the oxidation of the amine group, which was followed by Phase II reactions including conjugation with glutathione, and direct conjugation of the parent compound with glucuronic acid and leucine. The finding that sulfamethoxazole may be directly conjugated with biomolecules in higher plants merits further investigation, as conjugates may become deconjugated upon ingestion (Sakamoto et al., 2002). Therefore, consideration of such conjugates may offer a more accurate assessment of risk for such chemicals, for example, through the dietary intake (Paltiel et al., 2016). The potential for deconjugation may be particularly significant for antibiotics, due to an increased prevalence of antibiotic-resistant bacteria that pose a severe threat to the effectiveness of treatment for infections in healthcare worldwide (Boucher et al., 2009; Levy and Marshall, 2004).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.07.094.

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